INTERNATIONAL STEM CELL INITIATIVE-2

Defined media Performance Studies

Protocols for

Participating Laboratories

Sponsored by The International Stem Cell Forum.

Notes for this edition

Text highlighted in RED indicates material that is not being supplied centrally

Text highlighted in YELLOW indicates amounts or dilutions critical to the media composition and should be checked

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Label the two 'test' cell lines used with the codes assigned to your lab in the table above. The H9 control cells should be labelled Z 8

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Overall Project Plan– see Table. 1, below, for overview.

The aim of the study is to assess the ability of selected defined media to maintain and allow proliferation of 2 human embryonic stem cell (hES) lines (to be decided by discussion between the participating laboratories and the coordinator) over prolonged (up to 10 passages) and arrive at conclusions of the suitability of the different media formulations to sustain hES lines and recommendations of which, if any, can be taken forward to a wider screen in a subsequent phase of the study; our goal is to choose two media that will be tested by all participants in second phase of this study.

Participants will plate out the 2 selected hES isolates in 10 different media /matrix conditions. The conditions will include a reference condition of mouse embryonic fibroblasts, KO-DMEM / SR / bFGF. Cultures will be monitored by morphology and cell number on a passage by passage basis. These studies should be carried out with continuous liaison with the coordinator. The media will be provided and paid for by the ISCI project.

At selected passages, namely 0, 5and 10, cell cultures will be assessed by surface antigen expression by flow cytometry using 10 antibodies supplied by the coordinator and a control cell lines F9 and 2102ep (also supplied by the coordinator).

The table below shows an indicative summary of the activities to be carried out, however, these may be reviewed by discussion with the coordinator

Summary of Phase One of the Project.

Passage	Activity	Comment
0	Provide Karyotype data	Karyotype as close to start of study as posisble
	Photomicrographs of typical cell cultures	
	Cell counts to assess growth	ISCI2/SOP/007
	Flow Cytometry data of starting cultures	ISCI2/SOP/004
1	Transfer of cells to specific feeder-free conditions to be analysed. Photograph all cultures that are subsequently to be analysed	
	Cell counts to assess growth	ISCI2/SOP/007
2	Monitor morphology- photograph typical atypical colonies	
	Cell counts to assess growth	ISCI2/SOP/007
3	Monitor morphology- photograph typical atypical colonies	
	Cell counts to assess growth	ISCI2/SOP/007
4	Monitor morphology- photograph typical atypical colonies	
	Cell counts to assess growth	ISCI2/SOP/007
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	Flow Cytometry analysis	ISCI2/SOP/004
6	Monitor morphology- photograph typical atypical colonies Cell counts to assess growth	ISCI2/SOP/007
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8	Monitor morphology- photograph typical atypical colonies Cell counts to assess growth	ISCI2/SOP/007
9	Monitor morphology- photograph typical atypical colonies Cell counts to assess growth	ISCI2/SOP/007
10	Flow Cytometry analysis Cell counts to assess growth	ISCI2/SOP/004 ISCI2/SOP/007

Sample Coding.

All samples should be assigned a unique sample code based on the scheme listed below. Each lab has been assigned a code unique to them. Each labs 'test' cell lines have been assigned a unique letter with the H9 control cells being designated 'Z' for all labs.

For example

D-A-1-001-R-1

Is a sample from the first test cell line, growing in media one, prepared for RNA at passage one at the Karolinska Institute

Lab	Cell Line	Media	Sample	Type	Passage
D	A/B/Z	1	001	R (RNA)	1
B	C/D/Z			D (Meth)	
G	E/F/Z			S (SNP)	
MM	G/H/Z	10	999	K (K /type)	10

Lab Coding

Lab		**Lab Code**
KYOU	Kyoto U	B
KLNI	Karolinska	D
WCEL	WiCell	G
KUSC	USC	MM

Cell Line Codes

Label the two 'test' cell lines used with the codes assigned to your lab in the table above. The H9 control cells should be labelled Z

Media Code

Media 1 (Li et. al., 2005)
Media 2 (Liu et. al., 2006)
Media 3 (Vallier et. al., 2005)
Media 4 (Lu et. al., 2005)
Media 5 (Yao et. al., 2006)
Media 6 (Itoshi)
Media 7 (mTeSR)
Media 8 (STEM-PRO)
Media 10 (Control)

ISCI2/SOP/001 –Preparation of Feeder Cultures of Primary Mouse Embryonic Fibroblasts cells (ISCI Standard Conditions)

Purpose:

To prepare cultures of mouse embryonic fibroblasts for use as feeder cells for the culture of human embryonic stem cells.

Items required:

- PBS (Ca^{2+}, Mg^{2+} free)
- Pipettes, 5ml and 10ml
- Class II Microbiological Safety Cabinet (MSC)
- Cell culture microscope
- 15ml centrifuge tubes
- Centrifuge (refrigerated)
- CO_2 incubator
- MEFs
- **Media** (DMEM/F12 + 5% FCS+ 1% Glutamax)
- **Freezing Medium:** 90% foetal calf serum:10% DMSO (Sigma; cat no D-8779)
- **Gelatin**
- **Mitomycin C**

Notes:

- This protocol assumes that hES cells are grown in 25cm^2 flasks. Other culture vessels may be used; however the volume of medium per vessel may need to be adjusted.

Procedure:

Thawing:

1. Remove vial from liquid nitrogen and slightly loosen cap to allow trapped nitrogen to escape.

2. Thaw by immersing the bottom half only of the cryovial in a 37°C water bath and swirl the tube around. Do not immerse the whole tube in the water bath as this can lead to contamination problems.

3. Gently transfer the contents of the cryovial to a 15 ml centrifuge tube and then slowly add 10ml of DMEM/FCS.

4. Spin down at 200 g for 3 minutes.

5. Remove supernatant and then gently flick the bottom of the tube to disperse the pellet.

Resuspend in 10 ml DMEM/FCS and transfer to a fresh T75 tissue culture flask.

6. Add an additional 5 ml DMEM/FCS and transfer flask to incubator.

Passage of MEFs.

1. Aspirate medium from flask and wash cells once with PBS (w/o Ca^{++}, Mg^{++}).

2. Add 1ml Trypsin:EDTA per T75 flask and incubate at 37°C for 5 minutes.

3. Tap flask to dislodge cells and break up clumps. Add 9 ml DMEM/FCS and aspirate to form a single cell solution and then transfer to a 15 ml centrifuge tube.

4. Spin down at 200 g for 5 minutes.

5. Remove supernatant and gently flick the bottom of the tube to disperse pellet.

6. Add 10 ml of DMEM/FCS and then distribute equally between fresh T75 flasks. We typically

split MEFs at a 1:3 ratio. Place in 37°C incubator at 5% CO_2.

- **Notes:**For consistency and convenience, multiple flasks of MEFs can be amalgamated after trypsinisation and then distributed at a constant ratio of one T75 per cryovial.
- Appropriate precautions should be taken when handing liquid nitrogen, cryovials can explode when removed from liquid nitrogen, therefore face protection and gloves should be employed.
- Do not use MEFs past passage 4-5 as the MEFs begin to senesce after this stage.

Mitomycin C inactivation of MEFs

CAUTION!! Mitomycin-c is **harmful**. Use gloves and other appropriate protection when handling

Mitomycin-c. Dispose of Mitomycin-c solutions as hazardous waste.

1. Remove medium from a flask of MEFs (approx. 70% confluent) and replace with enough Mito-c-DMEM/FCS to cover cells in a layer a few millimetres deep.

2. Place in a 37°C incubator for 2-3 hours.

3. Whilst the MEFs are incubating, pre-treat tissue culture plastic (6-well plates or T25s) by covering with a 0.1% gelatin solution for at least 1-2 hours.

4. Aspirate Mito-c-DMEM/FCS.

5. Wash 3 times with PBS.

6. Add 4 ml Trypsin:EDTA per T75 flask (1ml/T25) and incubate at 37°C for 5 minutes. Tap theflask to dislodge and detach MEFs.

7. Inactivate Trypsin:EDTA with 9ml DMEM/FCS, gently aspirate to disperse clumps, transfer to a 15 ml centrifuge tube and pellet by centrifugation at 200 g for 5 minutes.

8. Aspirate supernatant and resuspend pellet in 10 ml DMEM/FCS for ease of counting.

9. Remove the gelatin solution from the tissue culture plates or flasks.

10. Count MEFs with a haemocytometer and seed out at 1.5 x 10_5 cells per T25* in total of 3mL.

11. The MEFs can be used after about 5-6 hours but are best left to settle overnight.

12. Use within one week.

* This density works well with the Wisconsin lines from Jamie Thomson's lab.

Freezing/ Using Frozen Inactivated MEFs

1. Inactivated MEFS can be frozen for later use. MEFs should be frozen in 10%DMSO, 90% FCS at approx $7x10^5$ cells per vial (for one T25).

2. Thaw by immersing the bottom half only of the cryovial in a 37°C water bath and swirl the tube around. Do not immerse the whole tube in the water bath as this can lead to contamination problems.

3. Gently transfer the contents of the cryovial to a 15 ml centrifuge tube and then slowly add 10ml of DMEM/FCS.

4. Spin down at 200 g for 3 minutes.

5. Remove supernatant and then gently flick the bottom of the tube to disperse the pellet.

6. Resuspend in 3 ml DMEM/FCS and transfer to a fresh gelatin coated T25 tissue culture flask.

7. The MEFs can be used after about 5-6 hours but are best left to settle overnight.

Solutions and Notes:

• Try to keep the MEFs actively dividing prior to mitotic inactivation, ideally by passaging the day before use.

• Do not use MEFs after passage 4-5.

• **Mito-c-DMEM/FCS:** Add 2 mg Mitomycin-c (Sigma; cat no M-4287) to 200 ml DMEM/FCS and sterilize by passing through a 0.2 µm cellulose acetate filter. Store at 4°C and use within 4 weeks.

• DMEM/FCS **must** be used to seed feeders. hES medium is **not suitable** for seeding out the feeders - the SR does not facilitate attachment of the feeders, nor will SR inactivate trypsin.

ISCI2/SOP/002 Passage of F9 Cell Line

Purpose:

To define the provedure for the growth and subculture of F9 cells.

Items Required

- PBS (Ca^{2+}, Mg^{2+} free)
- Pipettes, 5ml and 10ml
- Class II Microbiological Safety Cabinet (MSC)
- Cell culture microscope
- 15ml centrifuge tubes
- Centrifuge (refrigerated)
- CO_2 incubator
- MEFs
- **Media** (DMEM/F12 + 5% FCS+ 1% Glutamax)
- Gelatin

Notes

F9 expresses all the key surface antigens of differentiated cells detected by the antibodies included in the panel distributed for the ISCI2 (SSEA1). Therefore F9 cells are to be used as a standard and positive control showing differentiation for the immunofluorescence assays and FACs analysis.

1. Gelatin coat plates

   Thawing:

2. Remove vial from liquid nitrogen and slightly loosen cap to allow trapped nitrogen to escape.

3. Thaw by immersing the bottom half only of the cryovial in a 37°C water bath and swirl the tube around. Do not immerse the whole tube in the water bath as this can lead to contamination problems.
4. Gently transfer the contents of the cryovial to a 15 ml centrifuge tube and then slowly add 10ml of DMEM/FCS.
5. Spin down at 200 g for 3 minutes.
6. Remove supernatant and then gently flick the bottom of the tube to disperse the pellet.
7. Resuspend in 10 ml DMEM/FCS and transfer to a fresh T75 tissue culture flask.
8. Add an additional 5 ml DMEM/FCS and transfer flask to incubator.

Passage of F9s.

1. Aspirate medium from flask and wash cells once with PBS (w/o Ca^{++}, Mg^{++}).

2. Add 1ml Trypsin:EDTA per T75 flask and incubate at 37°C for 5 minutes.

3. Tap flask to dislodge cells and break up clumps. Add 9 ml DMEM/FCS and aspirate to form a single cell solution and then transfer to a 15 ml centrifuge tube.

4. Spin down at 200 g for 5 minutes.

5. Remove supernatant and gently flick the bottom of the tube to disperse pellet.

6. Add 10 ml of DMEM/FCS and then distribute equally between fresh gelatin coated T75 flasks. F9s are typically split F9s at a 1:10-1/20 ratio. Place in 37°C incubator at 5%CO_2.

ISCI2/SOP/003 – Passage of 2102Ep Human EC Cell Line

Purpose:

To define the procedure for the growth and subculture of 2102Ep cells.

Items required:

- Frozen ampoule of 2102Ep cells
- 2102Ep growth medium – DMEM/F12 + 10% FCS + 1% glutamine
- PBS (Ca^{2+}, Mg^{2+} free)
- Trypsin: EDTA
- Pipettes, 5ml and 10ml
- Cell culture flasks, 25cm^2 and 75cm^2
- Class II Microbiological Safety Cabinet (MSC)
- Cell culture microscope
- 15ml centrifuge tubes
- Centrifuge
- CO_2 incubator

Notes:

- 2102Ep is a nearly nullipotent human EC cell line which is easy to culture in an undifferentiated state, if maintained at high cell density. It expresses all the key surface antigens of human ES cells detected by the antibodies included in the panel distributed for the ISCI. If grown at low cell densities, it undergoes limited differentiation and expresses SSEA1, which is not expressed by undifferentiated hES cells. Therefore 2102Ep cells are to be used as a standard and positive control for pluripotency markers in the immunofluorescence assays and FACs analysis.

- Using the protocol detailed below, the cultures should reach confluence and be ready for passage in 3-5 days, with a yield of about 2 x 10^7 cells per 75cm^2.
- 2102Ep cells should only be cultured at high density (undifferentiated conditions) should express the surface antigens SSEA3, SSEA4, TRA-1-60, GCTM2, Thy1, TRA-2-54, CD9. A minority of cells should express SSEA1.

Procedure:

1. *Establishing 2102Ep cultures from frozen stocks.*

1.1. Rapidly thaw one ampoule of cells.

1.2. Transfer to a centrifuge tube and slowly add 5 – 10ml growth medium.

1.3. Centrifuge at 200 x g for 5 min. Discard supernatant.

1.4. Resuspend the cell pellet in 10ml growth medium and transfer to 2x25cm^2 flasks.

1.5. Incubate under a humidified atmosphere of 5% CO_2 in air at 37ºC, until the cells are confluent.

2. *To passage cells at high density (undifferentiated condition).*

2.1. Aspirate medium from flask and wash cells with PBS.

2.2. Add 1ml Trypsin-EDTA per flask and incubate at 37°C for 5 minutes.

2.3. Tap flask to dislodge cells and break up any clumps.

2.4. Resuspend the cells in 9ml growth medium.

2.5. Transfer to a 15ml centrifuge tube and centrifuge at 200 x g for 5 minutes.

2.6. Resuspend the pelleted cells in 10 ml medium and count the number of cells.

2.7. Seed the cells into flasks at a density of 5 $x10^6$ cells per 75cm^2, with 15 ml medium.

2.8. Incubate under a humidified atmosphere of 5% CO_2 in air at 37ºC.

ISCI2/SOP/004 – Undifferentiated hES Cell Surface Antigen Assay – FACS

Purpose:

To define the procedure for the preparation of hES cells for cell surface antigen analysis using FACS.

Items required:

- Trypsin: EDTA solution
- hES growth medium – see Appendix 1
- Wash buffer – PBS (Ca^{2+}, Mg^{2+} free) with 5% FCS and 0.1% sodium azide
- PBS (Ca^{2+}, Mg^{2+} free)
- Primary antibodies
- Secondary antibody
- Healthy hES cell cultures
- Haemocytometer
- Centrifuge
- 15ml centrifuge tubes
- Round bottom 96 well plate
- Plate seals
- Flow cytometer

Notes:

- The cells to be assayed for these surface antigens should be from healthy stock cultures.
- High and low density 2102Ep cells should be assayed with test samples to provide a standard and a positive control for the antibodies provided.
- Feeder cells should also be assayed with test samples, provided that you have enough of the primary antibodies to do this (200µl will be needed for this).

- This protocol assumes that the procedure is carried out using a 96 well plate; however other suitable plates/tubes may be used.
- Precise protocols for immunofluorescence analyses vary between labs. The following procedure is the one recommended for use in the ISCI, to help ensure comparability. It may be that you need to change the staining or analysis protocol used in your laboratory. If you believe that the protocol used in your laboratory is significantly different from the protocol set out below, please discuss this with the co-ordinator.
- ***Any deviations from standard protocols should be recorded on ISCI2/FRM/001 – Deviations from Standard Protocols.***

Table of Antigens to be Used

				Expected 2102Ep expression	
Antigen	Antibody	Species	Class	High Density	Low Density
Undifferentiated ES Cell Markers					
SSEA3	MC631	Rat	IgM	Positive	Positive
SSEA4	MC813-70	Mouse	IgG	Positive	Positive
TRA-1-60	TRA-1-60	Mouse	IgM	Positive	Positive
GCTM2	GCTM2	Mouse	IgM	Positive	Positive
L-ALP	TRA-2-54	Mouse	IgG	Positive	Positive
CD90(Thy-1)	Anti-Thy-1	Mouse	IgG	Positive	Positive
CD9	Anti-CD9	Mouse	IgG	Positive	Positive
Differentiation Markers					
SSEA1	MC480	Mouse	IgM	Negative	Positive
Pan human antigens					
TRA-1-85(0K(a))	TRA-1-85	Mouse	IgG	Positive	Positive
Control antibody					
P3X	Control IgG	Mouse	IgG	Negative	Negative

All the primary antibodies are monoclonals. Suggested dilutions of antibodies will be supplied when dispatched.

The secondary antibody provided is FITC tagged and recognizes mouse IgM and IgG.

Procedure:

1. Harvest hES cells by washing with PBS and then incubating with 1ml Trypsin: EDTA per 25cm^2 flask for 3 – 5 mins at 37^{o}C.

2. When cells begin to detach, add 9ml hES growth medium per ml Trypsin: EDTA.

3. Gently pipette to form a single cell suspension, and then count the cells using a haemocytometer.

4. Transfer the cells to a centrifuge tube and centrifuge at 200 x g for 5 minutes.

5. Resuspend cells in Wash Buffer to 2 x 10^6 cells per ml.

6. Distribute the antibodies at 50µl per well of a round bottom 96 well plate, two wells for each assay point (.***Primary antibodies are used as specified by the ISCI2 co-ordinator)*** To prevent carry-over from one well to another, it is good practice to arrange to use alternate wells of the plate.

7. Add 50µl of cell suspension (i.e. 10^5 cells) to each 50µl of antibody (at recommended dilution) in the wells of the 96 well plate.

8. Seal the plate by covering with a sticky plastic cover, ensuring that each well is sealed.

9. Incubate at 4^{o}C, ideally with gentle shaking, for 30 minutes.

10. Centrifuge the plate at 280 x g for 3 minutes, using microtitre plate carriers in a suitable centrifuge.

11. Dilute the secondary antibody 1/100 in wash buffer.

12. Check that the cells are pelleted, and then remove the plastic seal using a sharp motion, but holding the plate firmly, to avoid disturbing the cell pellet.

13. Discard the supernatant. The cells remain as pellets at the bottom of the wells.

14. Wash the cells by adding 100µl wash buffer to each well. Seal and agitate to re-suspend the cells. Centrifuge, as above.

15. Discard the supernatants and repeat for 2 further washes & spins.

16. After the third wash, discard the supernatants and add 50µl of the secondary antibody to each well.

17. Seal the plate, as above, and incubate with gentle shaking for 30 minutes at 4^{o}C.

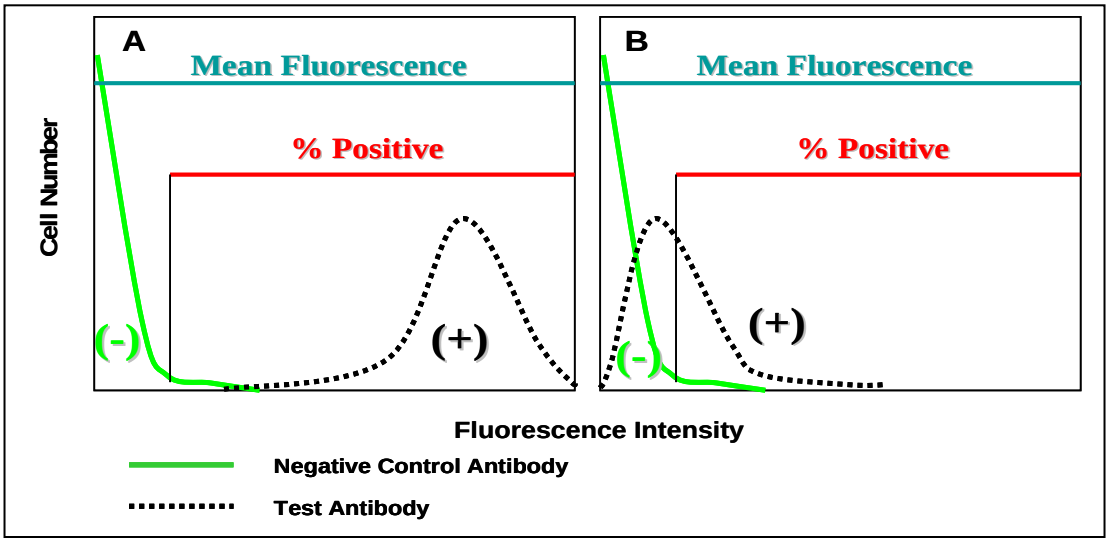
18. Centrifuge the cells and wash 3 times, as before.

Data Analysis and Reporting:

A negative control first antibody is provided and should be used for each cell line. This should be used to set a threshold for scoring the proportion of cells that are 'positive' for each test antigen. Typically this threshold should be set at the inflexion point on the negative control histogram, such that only 1% - 5% of the cells stained with the negative control would be scored 'positive'. Using this threshold, the % cells scored positive with each antibody should be recorded (see Figure below).

A second region should be set on the histogram, encompassing all the cells – from this gate the 'mean fluorescence intensity' of the cells stained with each antibody should be recorded (see Figure below). Although this value is arbitrary, and depends on local machine settings, inclusion of the 2102Ep standard cells will permit some comparability between the results from different labs.

Figure: Immunofluorescence Histograms



Note:

In Panel A, the positive antibody stains cells substantial brighter than the negative control; probably nearly 100% of the cells would be scored as antigen positive. In Panel B, the positive histogram overlaps significantly with the negative control histogram; rather less than 100% of cells will be scored positive for this antigen. The mean fluorescence of cells stained with the test antibody in Panel B will be substantially less than the mean fluorescence of the cells stained with the test antibody in Panel A. The two numbers, mean fluorescence and % positive cells, provide two complementary numbers for comparing results between cells, antibodies and laboratories.

Please record the % cells positive and mean fluorescence of each cell line for each antibody, ensuring that data for 2102Ep cells, and for negative control antibodies for each cell line are reported. In addition can participating laboratories where possible save and archive raw data from the flow cytometric analysis as so called 'listmode' data . Any queries regarding flow cytometry and data recording can be directed to Mark.Jones@ sheffield.ac.uk

ISCI2/SOP/007 – Counting of cells to assess Growth Characteristics of hES cell lines

Purpose: To acquire an accurate cell counts

.

Items Required:

- Healthy hES cultures
- Hemocytometer
- Tally Counter
- 4% Trypan Blue Solution
- PBS (no Mg++, Ca ++)
- Trypsin /EDTA solution (or similar cell dissociation solution)
- hES media

Notes:

Non-viable cells will appear as dark stained cells without a refractile edge

Procedure:

Trypsinization of hES Cell Cultures

1. Harvest hES cells by washing with PBS and then incubating with 1ml Trypsin: EDTA per 25cm^2 flask for 3 – 5 mins at 37^{o}C.

2. When cells begin to detach, add 9ml hES growth medium per ml Trypsin: EDTA.

3. Gently pipette to form a single cell suspension.

4. Transfer 0.1ml of the single cell suspension to a fresh sterile Eppendorf.

5. Add 0.1ml of 0.4% Trypan Blue. Mix by gentle pipetting.

Counting of a Single Cell Suspension using a Hemocytometer.

6. Clean Hemocytometer coverslip and slide with 70% absolute ethanol and dry.

7. Place the coverslip centrally on the etched section of the hemocytometer slide.

8. Using a pulled glass pasteur pipette or a disposable 'Gilson' tip add to the hemocytometer just enough cell suspension to flood the gridded surface.

9. Focus on a single sub-grid using a magnification that allows only one sub-grid to be visible in the field of view at one time. This will usually be with a 10x objective. The sub-grids are bounded by triple-ruled lines and lie at the corners of the hemocytometer. Count at least 4 sub-grids and average to generate cell count.

10 Cell number per mL can be calculated by the following equation:

$$\frac{\text{(Dilution factor of cells:trypan blue).e.g (2xTotal cell count) x}10^4}{\text{(Number of grids counted)}} \qquad \text{e.g. } 4$$

ISCI2/SOP/009 – Coating Plates with Geltrex

Items Required

- Geltrex™
- DMEM/F-12
- Parafilm
- Suitable pipettes
- Culture dishes (35mm or 60mm)
- 50mL conical tubes

1. Thaw a whole bottle (5 mL) of Geltrex™ at **2-8°C overnight**.

2. On the next day, promptlydispense in 1 mL aliquots of stock solution to pre-chilled, 50 mL conical tubes and store at -20°C.

3. At time of dish coating, thaw 1 tube (1mL) slowly at 2-8°C and add 29 mL D MEM/F-12 to each tube. Cover the whole surface of the culture plate with working solution (1 mL for 35 mmdish, 1.5 mL for 60 mm dish).

4. Seal each dish with parafilm to prevent dish from drying out and incubate 1 hour at room temperature. Store at 2-8°C; dish can be used up to 1 month.

3. At time of use, aspirate Geltrex™ solution and immediately plate cells in pre-equilibrated complete medium. Do not allow surface to dry out.

ISCI2/SOP/010 - Protocol For Harvesting Metaphases From Stem Cells

Purpose

To prepare cells for karyotyping by G-Banding

Items required

REAGENT	SUPPLIER	CATALOGUE NUMBER
Colcemid 10µg/ml (Karyomax) (diluted to 2µg/ml with PBS)	GIBCO	15212-046
Trypsin	GIBCO	27250-018
Gurr's buffer pH6.8	GIBCO	10582
Methanol (AnalaR)	BDH (VMR International)	101586B
Acetic acid (AnalaR)	BDH (VMR International)	100015N
KCl (AnalaR)	BDH (VMR International)	101983K
Glass microscope slides	BDH (VMR International)	631-0112
Leishmann's/Giesma Stain	Sigma	L6254-25G
DAPI	Vector Laboratories	H-1200

- **Small (less than 10mLs) conical bottomed test tube**
- **Appropriate pipettes**

- **Centrifuge**

Additional Optional Equipment

Fluorescent microscope.

Inverted phase microscope

Light microscope

Notes

- This protocol works best for cells that are approximately 3-5 days post subculture and have recently been "fed". (i.e. in exponential phase of growth to promote higher numbers of cells in metaphase) .
- Typically cultures should be grown in T25 flasks with approximately 10mls of media to produce suitable numbers of cells in metaphase.

Procedure

1. Add 0.4ml of 2µg/ml colcemid (an agent that promotes mitotic arrest) to a 10ml culture (or 0.2ml for a 5ml culture). Incubate for 4 hours at 37^{o}C.

2. Pour off the media into a test tube (conical bottomed and of less than 10mls volume) and centrifuge for 8 minutes at 1200rpm (80-100g). Remove the supernatant from the pellet of cells.

3. In the meantime, add 1ml of pre-warmed 0.25% trypsin/EDTA to the flask of cells and incubate for 5-10 minutes, until the cells have detached from the surface of the culture flask and from feeder cells.

4. Transfer all the trypsinised cells from the flask into the test tube from step 2 and transfer half of this suspension to a second test tube (splitting into two tubes should produce a cleaner preparation).

5. Add approximately 6mls of a pre-warmed (37^{o}C), hypotonic solution of 0.0375M potassium chloride (made up with distilled water) to each tube.

6. Incubate the tubes for 5 minutes at 37^{o}C.

7. Centrifuge at 1200rpm (80-100g) for 8 minutes.

8. Remove supernatant leaving about 0.5ml fluid in the tube and vigorously re-suspend cells.

9. Add about 4ml fixative (3:1 methanol : acetic acid). It is important that the first 2mls of fixative be added slowly, drop by drop whilst constantly agitating the tube as you do so (adding the fix too quickly will destroy cells). The remaining 2mls and subsequent fixations can be added normally.

10. Centrifuge at 1200rpm (80-100g) for 8 minutes.

11. Remove supernatant leaving about 0.5mls fluid in the tube and re-suspend cells.

12. Repeat steps 8-10 twice.
13. Resuspend pellet in 4mLs fixative

13 Label tube with passage level, date prepared and originating laboratory .Store in -20^{0}C freezer until needed. Forward on dry ice to Sheffield (address attached

Note

- Checking the success of the harvesting procedure can be done anytime after harvesting. Cells can be left at -20'C in the freezer for up to a year assuming there is enough fixative and there will not be any detrimental effects. Cell suspensions can be moved in and out of freezer regularly without any adverse effects.

In order to check that the harvest is successful it is necessary to examine a drop of the suspension on a standard microscope slide. (If the suspension appears slightly cloudy after the harvest then go straight to step 16).

14 Centrifuge one of the test tubes at 1200 rpm (80-100g) for 8 minutes.

15 Remove the supernatant but leaving at least 1ml and re-suspend cells. If the suspension is too thick it will appear white, in which case add extra fixative to dilute until the suspension is slightly cloudy.

16 Use a 2ml pipette to place one drop of suspension onto a clean microscope slide. Use blotting paper to remove excess liquid gently from the ends of the slide. Leave the slide on the work bench to dry.

17 Top up the suspension with fix and return to freezer.

Note

- There are several techniques that can be used to visualise the metaphases. Three methods are described below:

Alternative number 1:

Stain the slide with DAPI and examine under a fluorescent microscope (we use 20ul of mounting medium with DAPI in 1000ul of mounting medium for fluorescence – Vectasheild). See picture below for DAPI image.

Alternative number 2:

Examine the slide directly under an inverted phase microscope. See picture below for example image.

Alternative number 3:

1. Stain the slide with Leishmann's/Giesma (made up 1:4 in Gurrs buffer) for 1 minute.

2. Wash off with Gurrs (neutral) buffer.

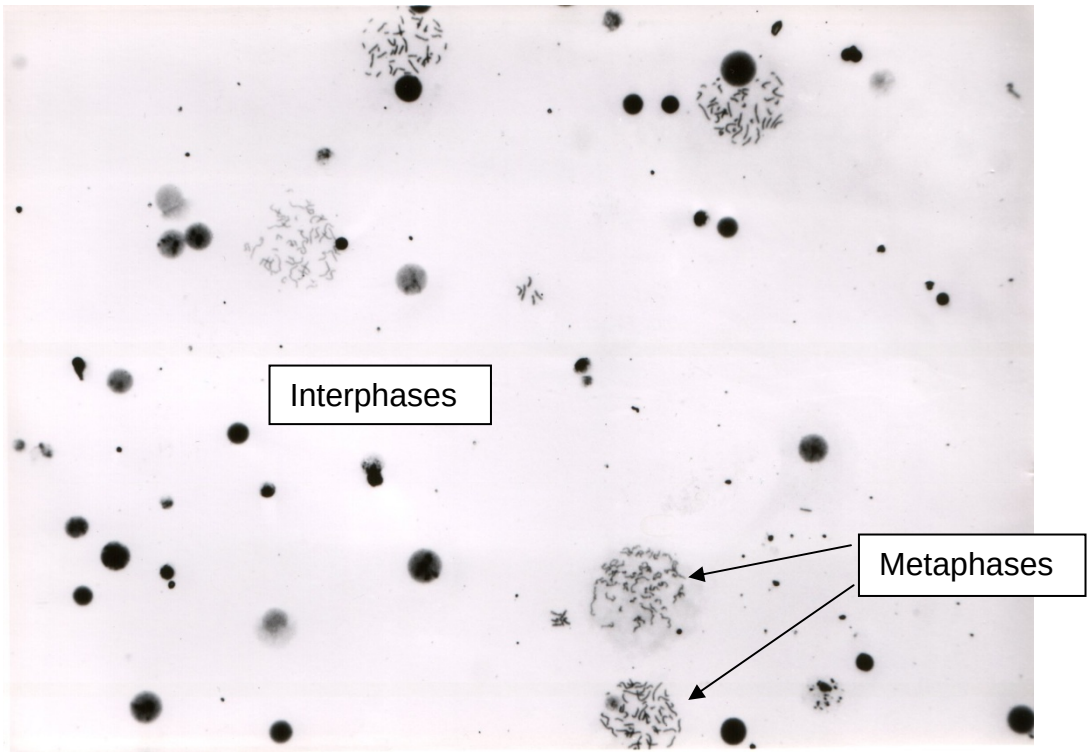
3. Examine slide under light microscope (see picture below).

Notes

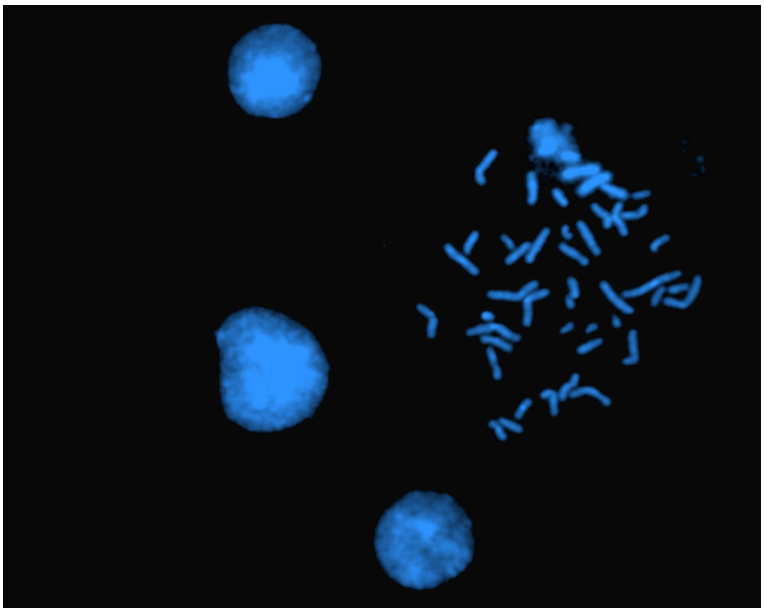
- On a slide the majority of cells should be seen as dark round interphase cells and a few metaphases. Metaphases will appear as a cluster of dark chromosomes (see example below).There might be as little as 5 metaphases per slide or up to 20 or more.

- If there are no metaphases visible then the suspension may be too dilute, in which case add fixative and repeat steps 13-15 with less dilution.

Alternative 2 and 3. Block stained Interphase and metaphase cells under a light microscope (100X magnification).



Alternative 1. Interphases and a metaphase cell stained with DAPI under a fluorescent microscope (400X magnification).



ISCI2/SOP/0011– Passage of hES cells (MEDIA 1, Li et. al., 2005)

Purpose:

To define the ISCI2 standard culture conditions for the subculture of human embryonic stem (hES) cells in this specific growth media.

Items required:

- Collagenase – or suitable alternative
- Media 1– see Appendix 1
- PBS (Ca^{2+}, Mg^{2+} free)
- Geltrex
- Pipettes, 5ml and 10ml
- Class II Microbiological Safety Cabinet (MSC)
- Cell culture microscope
- 15ml centrifuge tubes
- Centrifuge (refrigerated)
- CO_2 incubator
- DMEM/F12

Notes:

- This protocol assumes that hES cells are grown in 25cm^2 flasks. Other culture vessels may be used; however the volume of medium per vessel may need to be adjusted.

Procedure:

1. Using a microscope, select hES cells that are ready to passage.

2. Transfer flask to a Class II MSC and aspirate the spent medium from the flask.

3. Add 2ml of collagenase per 25cm^2 flask. Incubate the flask at 37°C for 8-10 minutes, until the edges of the hES colonies start to curl (as observed under the microscope).

4. Gently scrape the cells from the flask using a sterile 1000 µL pipette tip

5. Add 5ml hES medium and gently resuspend the cells.

6. Transfer suspension to a centrifuge tube. Centrifuge at 50 x g for 3 minutes at 4°C.

7. Using a plastic pipette, remove the supernatant from the hES cell pellet.

8. Gently flick the tube to disperse the pellet, and carefully resuspend the cells in an appropriate volume of hES medium, allowing 1ml of medium for each new culture to be set up (i.e. for a 1:4 split ratio, resuspend in 4ml of medium).

9. Using a plastic pipette, remove the DMEM/F12 from the Geltrex coated flasks.

10. Add 1ml of hES cell suspension to each Geltrex coated flask.

11. Add a further 4ml of hES medium per flask, and then carefully transfer to a 37°C incubator, maintaining an even distribution of cells across the flask..

12. Microscopically check the appearance of the cells each day, and feed with fresh hES medium daily, until the cells are ready to passage.

ISCI2/SOP/0012 – Passage of hES cells (MEDIA 2, Liu et. al., 2006)

Purpose:

To define the ISCI2 standard culture conditions for the subculture of human embryonic stem (hES) cells.

Items required:

- Collagenase – or suitable alternative
- Media 2– see Appendix 1
- PBS (Ca^{2+}, Mg^{2+} free)
- Geltrex
- Pipettes, 5ml and 10ml
- Class II Microbiological Safety Cabinet (MSC)
- Cell culture microscope
- 15ml centrifuge tubes
- Centrifuge (refrigerated)
- CO_2 incubator
- DMEM/F12

Notes:

- This protocol assumes that hES cells are grown in 25cm^2 flasks. Other culture vessels may be used; however the volume of medium per vessel may need to be adjusted.

Procedure:

1. Using a microscope, select hES cells that are ready to passage.

2. Transfer flask to a Class II MSC and aspirate the spent medium from the flask.

3. Add 2ml of collagenase per 25cm^2 flask. Incubate the flask at 37°C for 8-10 minutes, until the edges of the hES colonies start to curl (as observed under the microscope).

4. Gently scrape the cells from the flask using a sterile 1000 µL pipette tip

5. Add 5ml hES medium and gently resuspend the cells.

6. Transfer suspension to a centrifuge tube. Centrifuge at 50 x g for 3 minutes at 4°C.

7. Using a plastic pipette, remove the supernatant from the hES cell pellet.

8. Gently flick the tube to disperse the pellet, and carefully resuspend the cells in an appropriate volume of hES medium, allowing 1ml of medium for each new culture to be set up (i.e. for a 1:4 split ratio, resuspend in 4ml of medium).

9. Using a plastic pipette, remove the DMEM/F12 from the Geltrex coated flasks.

10. Add 1ml of hES cell suspension to each Geltrex coated flask.

11. Add a further 4ml of hES medium per flask, and then carefully transfer to a 37°C incubator, maintaining an even distribution of cells across the flask.

12. Microscopically check the appearance of the cells each day, and feed with fresh hES medium daily, until the cells are ready to passage.

ISCI2/SOP/0013a – Passage of hES cells (MEDIA 3, Vallier et al., 2005, Brons et al., 2007)

Purpose: The objective of this protocol is to establish conditions for growing human Embryonic Stem Cells (hESCs) in the absence of a feeder layer, by growing in conditioned medium, serum, no complex matrices in order to establish simplified conditions for analyzing the mechanisms of hESCs pluripotency and differentiation.

Overview: This protocol outlines conditions for growing hESCs in CDM, starting from conventional cultures on feeder layers. Two variations are provided for the type of Matrix used: Foetal Bovine Serum (FBS) pre-coating or Fibronectin pre-coating of plates. ISCI2/SOP/0013a should be initially followed (i.e. coating of plates with BSA), however, if problems arise plates should be coated with Fibronectin (ISCI2/SOP/0013b). This must be noted on the appropriate form.

Items Required

- Porcine gelatin (Sigma)
- Water (Sigma)
- MEF medium, (containing 10% FBS from Hyclone)
- Culture plate (6 well, 12 well or 10cm)
- 5% CO_2 incubator at 37^0C
- hESC growing on MEFs
- PBS
- Collangenase IV
- Pipettes
- CDM (Chemically Defined Medium)
- Activin A (RandD Systems 338-AC/CF)
- FGF2 (RandD Systems, 3718-FB/CF)

- Fibronectin (Chemicon FC010)
- Centrifuge
- Centrifuge tubes.
- BSA (Europa Bioproducts or Equitech-Bio Inc)

Protocol for FBS coated plates.

Precoating with FBS

1- Precoat plates with Porcine gelatin (Sigma) 0.1% in embryo tested water (Sigma) for 15-60 minutes at room temperature.

2- Precoat plate with MEF medium containing 10% FBS (Hyclone) for 24 hours-7 days at 37C incubator 5% CO2.
1 ml MEF medium / well 6 wells plate
6 ml MEF medium / 10 cm plates

Transfer from feeder layer.

3- Wash hESCs colonies once in PBS and then add 3ml collagenase IV 1 mg/ml per 60mm plate. Incubate 15 minutes at 37C. Scrape the colonies with a 5 ml pipette and dissociate them into clumps by pipeting 1-4 times (try to generate big clumps).

4- Gently resuspend the clumps in 3 ml of CDM (without Activin or FGF).

5- Transfer suspension to a centrifuge tube. Centrifuge at 50 x g for 3 minutes at 4°C.

6- Gently flick the tube to disperse the pellet and resuspend the clumps in appropriate amount of CDM containing 10 ng/ml Activin A (RandD Systems 338-AC/CF) + 12 ng/ml FGF2 (RandD Systems, 3718-FB/CF). Suggested plating

density: 1 confluent plate (p60mm) of hESCs grown on feeders into 6-12 wells of 6 well plate in CDM + Activin + FGF, therefore resuspend cells in 1mL per well of a 6 well plate.

7- Wash the FBS pre-coated plate once with PBS. 2ml PBS / well 6 wells plate

8- Plate the clumps at low density (100-300 clumps/well) on a FBS-coated 6 wells plate in 2mL CDM then carefully transfer to a 37^0C incubator at 5%CO_2 ensuring that colonies are evenly distributed on the plate.

9- Leave 5-7 days until the colonies reach a **large size** (4 to 6 times bigger than colonies grown on feeder).

Note: Cells in CDM will only grow at 5% CO_2 (not at 10% $CO_{2)}$.

Splitting in CDM+Activin+FGF.

10- Wash the cells once in PBS and then add 1.5 ml collagenase IV 1 mg/ml for 10-15 minutes at room temperature. Scrape the colonies with a 5 ml pipette and dissociate them into smaller clumps by pipeting gently 1-2 times (try to generate big clumps).

11- Gently resuspend the clumps in 3 ml of CDM (without Activin or FGF).

12- Transfer suspension to a centrifuge tube. Centrifuge at 50 x g for 3 minutes at 4°C.

13- Gently flick the tube to disperse the pellet and resuspend the clumps in appropriate amount of CDM containing 10 ng/ml Activin A (RandD Systems 338-AC/CF) + 12 ng/ml FGF2 (RandD Systems, 3718-FB/CF).

14- Plate the dissociated colonies on 4-5 new FBS coated plates at low density. .

ISCI2/SOP/0013b

Protocol with Fibronectin coated plates.

Precoating with human Fibronectin

1- Add 1.5 ml PBS per well of a 6 wells plate. Then add 15 µl of Fibronectin 10 mg/ml (Chemicon FC010) per well. For a 12 well plate add 0.5 ml PBS per well and then 5 µl of Fibronectin.
Incubate at 4C overnight or 30 min /1 hour at 37C.

Transfer from feeders.

2- Wash the cells once in PBS and then add 3ml collagenase IV 1 mg/ml per 60mm plate. Incubate 15 minutes 37C. Scrap the colonies with a 5 ml pipette and dissociate 0-3 times (try to generate big clumps).

3- Gently resuspend the clumps in 3 ml of CDM (without Activin or FGF).

4- Transfer suspension to a centrifuge tube. Centrifuge at 50 x g for 3 minutes at 4°C.

5- Gently flick the tube to disperse the pellet and resuspend the clumps in appropriate amount of CDM containing 10 ng/ml Activin A (RandD Systems 338-AC/CF) + 12 ng/ml FGF2 (RandD Systems, 3718-FB/CF)

6- Wash the Fibronectin coated plate once with PBS.

7- Plate the clumps at low density (100-300 clumps/well) on a fibronectin-coated 6 wells plate in CDM containing 10ng/ml Activin A + 12ng/ml FGF2 .
Typical plating is one confluent p60mm of hESCs grown on feeder in 6-8 wells of 6 well plate.

8- Leave 5-7 days until the colonies reach a **very large size** (4 to10 times bigger than colony grown on feeders)**.**

Splitting in CDM/AF medium.

9- For splitting. Wash colonies once with PBS and then add 1.5 ml of collagenase IV 1 mg/ml for 10 minutes at room temperature. Scrape the colonies with a 5 ml pipette and dissociate the cells into clumps by pipeting gently 1-2 times (Big clumps are definitively better).

10- Gently resuspend the clumps in 3 ml of CDM (without Activin or FGF).

11- Transfer suspension to a centrifuge tube. Centrifuge at 50 x g for 3 minutes at 4°C.

12- Gently flick the tube to disperse the pellet and resuspend the clumps in appropriate amount of CDM containing 10 ng/ml Activin A (RandD Systems 338-AC/CF) + 12 ng/ml FGF2 (RandD Systems, 3718-FB/CFPlate the dissociated colonies on 3-4 new Fibronectin coated plates at low density .

Trouble Shooting

It may be necessary to optimize this SOP depending on the hESC cell-line being cultured. Below are a number of suggested variations that may improve the culture of hESC cells using the above method.

Please Note: Any Variations from this SOP must be recorded on the correct form.

-**Differentiation and production of stroma cells** may be observed, this can be limited by increasing the quanitity of FGF and Fibronectin levels.

It may be necessary to test several doses of Activin/FGF/Fibronectin.

- Adhesion: Attachment problem of hESC in CDM+Activin+FGF (especially with Fibronectin coated plates) may be observed. If EB-like colonies are observed the day after passaging,cells should be left an additional day before changing the medium. Usually, the colonies spread during the second night after passaging.

Variation in batches of BSA may be responsible for this problem therefore it may be necessary to change the batches used. Increasing or decreasing dose of Fibronectin / PBS used to coat plates may improve the attachment of the clumps.

Increasing time of Collagenase incubation or using Dispase may be necessary..

- From feeders: hESCs grown on feeders can be difficult to transfer into CDM+Activin+FGF medium, high quality hESC should be used. Manual cutting of colonies may be necessary for the first few passages when transferring cells from feeders to feeder free.

ISCI2/SOP/0014 – Passage of hES cells (MEDIA 4, Lu et. al., 2005)

Purpose:

To define the ISCI standard culture conditions for the subculture of human embryonic stem (hES) cells.

Items required:

- Collagenase – or suitable alternative
- Media 4– see Appendix 1
- PBS (Ca^{2+}, Mg^{2+} free)
- Geltrex
- Pipettes, 5ml and 10ml
- Class II Microbiological Safety Cabinet (MSC)
- Cell culture microscope
- 15ml centrifuge tubes
- Centrifuge (refrigerated)
- CO_2 incubator
- DMEM/F12

Notes:

- This protocol assumes that hES cells are grown in 25cm^2 flasks. Other culture vessels may be used; however the volume of medium per vessel may need to be adjusted.

Procedure:

1. Using a microscope, select hES cells that are ready to passage.

2. Transfer flask to a Class II MSC and aspirate the spent medium from the flask.

3. Add 2ml of collagenase per 25cm^2 flask. Incubate the flask at 37°C for 8-10 minutes, until the edges of the hES colonies start to curl (as observed under the microscope).

4. Gently scrape the cells from the flask using a sterile 1000 µL pipette tip

5. Add 5ml hES medium and gently resuspend the cells.

6. Transfer suspension to a centrifuge tube. Centrifuge at 50 x g for 3 minutes at 4°C.

7. Using a plastic pipette, remove the supernatant from the hES cell pellet.

8. Gently flick the tube to disperse the pellet, and carefully resuspend the cells in an appropriate volume of hES medium, allowing 1ml of medium for each new culture to be set up (i.e. for a 1:4 split ratio, resuspend in 4ml of medium).

9. Using a plastic pipette, remove the DMEM/F12 from the Geltrex coated flasks.

10. Add 1ml of hES cell suspension to each Geltrex coated flask.

11. Add a further 4ml of hES medium per flask, and then carefully transfer to a 37°C incubator, maintaining an even distribution of cells across the flask r.

12. Microscopically check the appearance of the cells each day, and feed with fresh hES medium daily, until the cells are ready to passage.

ISCI2/SOP/015 – Passage of hES cells (MEDIA 5, Yao et. al., 2006)

Purpose:

To define the ISCI2standard culture conditions for the subculture of human embryonic stem (hES) cells.

Items required:

- Collagenase – or suitable alternative
- Media 5– see Appendix 1
- PBS (Ca^{2+}, Mg^{2+} free)
- Geltrex coated plates
- Pipettes, 1ml, 5ml and 10ml
- Class II Microbiological Safety Cabinet (MSC)
- Cell culture microscope
- 15ml centrifuge tubes
- Centrifuge (refrigerated)
- CO_2 incubator
- DMEM/F12

Notes:

- This protocol assumes that hES cells are grown in 25cm^2 flasks. Other culture vessels may be used; however the volume of medium per vessel may need to be adjusted.

Procedure:

1. Using a microscope, select hES cells that are ready to passage.

2. Transfer flask to a Class II MSC and aspirate the spent medium from the flask.

3. Add 2ml of collagenase per 25cm^2 flask. Incubate the flask at 37°C for 8-10 minutes, until the edges of the hES colonies start to curl (as observed under the microscope).

4. Gently scrape the cells from the flask using a sterile 1000 µL pipette tip

5. Add 5ml hES medium and gently resuspend the cells.

6. Transfer suspension to a centrifuge tube. Centrifuge at 50 x g for 3 minutes at 4°C.

7. Using a plastic pipette, remove the supernatant from the hES cell pellet.

8. Gently flick the tube to disperse the pellet, and carefully resuspend the cells in an appropriate volume of hES medium, allowing 1ml of medium for each new culture to be set up (i.e. for a 1:4 split ratio, resuspend in 4ml of medium).

9. Using a plastic pipette, remove the DMEM/F12 from the Geltrex coated flasks.

10. Add 1ml of hES cell suspension to each Geltrex coated flask.

11. Add a further 4ml of hES medium per flask, and then carefully transfer to a 37°C incubator, maintaining an even distribution of cells across the flask.

12. Microscopically check the appearance of the cells each day, and feed with fresh hES medium daily, until the cells are ready to passage.

ISCI2/SOP/016 – Passage of hES cells (MEDIA 6,hESF9 (alt name HESF-GRO))

Purpose:

To define the ISCI2standard culture conditions for the subculture of human embryonic stem (hES) cells.

Items Required.

- hESF9 medium (Cell Science & Technology Institute, Inc. Japan) http://www.cstimedia.com/
- Type IA Collagen (Nitta gelatin. It is recommended that standard collagen preparations such as Sigma type I collagen are NOT used with this media). This will be supplied from Itochu as "CellMatrix"
- Ready-made 1x Dulbecco's Phosphate Buffered Saline (such as Sigma D8537, Do not use PBS in washed bottle. **Micro amounts of residual detergents can damage hES cells**.)
- EDTA/4NA (Ethylenediaminetetraacetic acid tetrasodium salt dihydrate, cell culture tested, Sigma E6511)
- Pipettes, 1ml, 5ml and 10ml
- Class II Microbiological Safety Cabinet (MSC)
- Cell culture microscope
- 15ml centrifuge tubes
- Centrifuge (refrigerated)
- CO_2 incubator
- $25cm^2$ flasks

- Foil or plastic wrap
- KSR based medium

1.Coating flasks with Collagen Type IA.

1. Cool PBS on ice.
2. Dilute 1ml of CellMatrix (type I collagen (Nitta gelatin)) with 9 ml cold PBS.
3. Mix the Solution by gentle pipetting , ensure the solution is mixed well.
4. Coat a 25cm^2 flask with 1ml of the diluted collagen solution.
5. Until the point of the use, wrap the flask with foil or plastic-wrap, ensure that the flask does not dry out.
6. Incubate the flask in 37ºC incubator for at least 3 hours.

2. Transfer human ES cells from the culture on feeder to the culture without feeder in hESF9 medium

It is preferable to use smaller colonies than are normally passaged in KSR/feeder based media.

Dissociate human ES cells cultured on feeders by your routine procedure

(1) Collect the cells by the general medium, such as KSR based medium into tube.
(2) Centrifuge at 50 x g for 3 minutes at 4°C
(3) Add 10ml hESF9 medium to the tube.
(4) Centrifuge at 50 x g for 3 minutes at 4°C
(5) Suspend all the cells in 8 mls.

(6) Seed the cells in 8 mls on a 25cm^2 flask coated with collagen*.

Do not use dried flask.

3. Medium change

On the next day of every subculture, change the medium.

(1) Calculate how much medium is need.

(2) Transfer the medium requirement to 50ml plastic tube*.

(3) Warm the medium.

(4) Remove all the medium and add 8ml of fresh and warm medium.

(5) After that, change the medium every 2 day.

*Do not warm the whole medium bottle.

****Do not reheat the medium.**

4. Passage

(1) Remove the medium.

(2) Add 1ml of 0.2% EDTA/4Na in PBS**.**

(3) Keep the flask in the room temperature for 20~30 seconds under the microscope to check the shape of colonies.

(4) When the edge of colonies starts to shrink, vigorously tap the sides of the flasks.

(5) Collect the detached colonies in 9 ml of cold hESF9 medium **quickly.**

(**Do not pipette the cell suspension at this point.**)

(6) Centrifuge at 50 x g for 3 minutes at 4°C.

(7) Remove the medium and add cold fresh hESF9 medium.

(8) Pipette **three times gently.**

(9) Centrifuge at 50 x g for 3 minutes at 4°C.

(10) Remove the medium and add 10 ml of cold fresh hESF9 medium.

(11) Split the cells 1:2 or 1:3 and seed the cells in 8 ml of hESF9 medium on a 25 cm^2 flasks coated with collagen.

Usually, the cells should be passaged on the second day after the first passage. After the second passage, the cells should be passaged every 5 days.

Trouble Shooting

If applicable please remember to note any deviations from the SOP on the relevant form.

The concentration of EDTA depends on cell lines. If the colonies fractionate into too small ones, please try 0.1% EDTA/4Na solution.

Some cell lines do not prefer EDTA.Collagenase should be used as an alternative usinf the following protocol:

(i) Remove the medium.

(ii) Add 1ml of 0.3% collagenase.

(iii) Keep the flak at the room temperature for 30 min.

(iv) Remove the collagenase.

(v) Add 1ml of 0.1% EDTA.

(vi) Keep the flak at the room temperature for 15~30 min.

(vii) Tap the flask.

(viii)Then, Collect the dissociated colonies/cells by the above method (see step5).

ISCI2/SOP/017 – Passage of hES cells (MEDIA 7, mTeSR)

Purpose:

To define the ISCI2 standard culture conditions for the subculture of human embryonic stem (hES) cells

Items Supplied

Component	Cat #	Vol/ml	Storage
mTeSR1 Basal medium	05851	400	2-8°C
mTeSR1 5X supplement	05852	100	-20°C

Items required:

- Dispase – or suitable alternative
- Media – see Appendix 1
- PBS (Ca^{2+}, Mg^{2+} free)
- Matrigel
- Pipettes, 1ml, 5ml and 10ml
- Class II Microbiological Safety Cabinet (MSC)
- Cell culture microscope
- 15ml centrifuge tubes
- Centrifuge (refrigerated)
- CO_2 incubator
- DMEM/F12

Procedure:

Preparation of mTeSR1

1. Thaw mTeSR1 5X supplement at Room temperature (15-25°C) OR 2-8°C overnight
2. Add 100 ml mTeSR1 5X supplement to 400 ml of mTeSR1 Basal medium, mix well

Notes

- Ensure that lot numbers of mTeSR1 5X supplement and mTeSR1 Basal medium end with the same letter.
- mTeSR 5X supplement can be dispensed into working aliquots and stored at -20°C for up to 6 months. Do not re-freeze, make up working media within 1 day of defrosting.
- Working mTeSR1 media is stable for 2 weeks at 2-8°C or 6 months at -20°C.

Passaging Human embryonic Stem Cells into mTeSR1

1. Aliquot the required amount of mTeSR1 and warm to room temperature. Recommended amounts are 2-2.5ml per well of a 6-well plate. Warm diluted (1mg /ml)dispase, PBSm DMEM/F12 to room temperature
2. Remove spent medium from hESC cultures and rinse (2mls /well) with PBS
3. Add pre-warmed dispase to colonies for 7 mins at 37°C. Colony edges should appear slightly curled but the colonies should still be attached
4. Remove Dispase, and gently rinse each well with 2ml DMEM/F12 2-3 times.
5. Add 2mls of mTeSR1 and scrape off cells with a cell scraper or serological pipette tip.
6. Transfer the detached cells to a 15ml conical tube, rinse the well with an additional 2mls of mTeSR1 to collect any remaining colonies and add to the conical tube.
7. Centrifuge the detached colonies at 300g for 5 minutes at room temperature.
8. Remove the supernatant and add 1-2 ml of TeSR1 per well required. Resupend the pellet gently with a P1000 micropipette (1-2 times).
9. Add required aliquot of cell suspension to a fresh matrix coated plate.

10. Move the plate on both its horizontal x and y axes in short, quick motions to disperse the colonies evenly

11. Return the plate to a 37°C, 5% CO2 incubator.

Notes:

- Colonies in mTeSR1 are passaged when large and beginning to merge. The centres of the colonies should be dense and phase-bright under low magnification phase contrast illumination. There is a 24hr window for passaging

- Cultures are usually passaged after 5-7 days post seeding.

- Colonies will appear transparent and loosely packed up to 4 days post seeding.
- Colonies passaged too frequently can have attachment problems.

ISCI2/SOP/018 – Passage of hES cells (MEDIA 8, STEM-PRO)

Materials Required

- Collagenase – or suitable alternative
- Wash Solution –See Appendix 1
- Media – see Appendix 1
- PBS (Ca^{2+}, Mg^{2+} free)
- Geltrex
- Pipettes, 5ml and 10ml
- Class II Microbiological Safety Cabinet (MSC)
- Cell culture microscope
- 15ml centrifuge tubes
- Centrifuge (refrigerated)
- CO_2 incubator
- 21½ gauge needle

Passaging Using Collagenase

1. Warm appropriate amount of 10 mg/mL Collagenase IV solution, complete medium and wash medium to 37^{o}C in a water bath. .

2. Set up hESC plate on a dissecting microscope in a bio-safety cabinet or laminar flow to comfortably observe colonies.

3. Cut out and remove any overtly differentiated colonies with a 21½ gauge needle.

4. Aspirate the medium and gently add 1-2 mL of collagenase.

5. Leave for a maximum of 3 minutes to dislodge cell colonies from substrate.

6. Remove collagenase, rinse with D-PBS, and then add 3 mL wash medium.

7. Gently scrape dish using a sterile 1000 µL pipette tip.

8. Gently transfer clumps using a 5 mL pipette and place into a 15 mL tube.

9. Wash plate with 3 mL of wash medium and add to tube.

10. Spin cells at 1000 x rpm (200 x g) for 2 minutes at room temperature.

11. Gently aspirate media and flick tube to loosen cells.

12. Gently resuspend the cells in pre-equilibrated complete medium using a 1 mL or 5 mL serological pipette.

13. Transfer cells to a Geltrex™-coated plate. See ISCI2/SOP/009 – Coating Plates with Geltrex

14. Mix plates gently to evenly spread out clumps and place the plate into an incubator set at 37OC with 5% CO2 in air.

15. Gently change media the next day to remove excess cells and provide fresh nutrients, and every day thereafter.

16. Observe cells every day and passage by the above protocol whenever required (approximately every 5-7 days).

NOTES

Important Guidelines for hESC Culture

In order to prevent differentiation and slow growth of hESCs grown in STEMPRO® hESC SFM, follow these guidelines:

• **Starter culture**: This must be a high quality culture, with a high density of cells, and primarily undifferentiated. The starter culture should be hESCs maintained on Matrigel™ or Geltrex™ in Mouse Embryonal Fibroblast-Conditioned Medium (MEF-CM), not on MEF feeder cells.

• **Passaging:** It is critical to achieve high plating/survival of colony pieces. The pieces must be a bit smaller than typical collagenase passaging on Matrigel™ or Geltrex™/ MEF-CM.

• Some **cell death** at passaging is normal, but not wide-scale cell death (i.e. <20% survival); this indicates poor passaging.

• **Timing of passaging**. Critical: the cultures need to grow to near-confluence, i.e., a day or two after the colonies are just touching, cultures should be harvested. This usually results in a cell density of 2.5 - 4 x 10^5 cells/cm^2 at time of harvest.

• Do not over-expose cells to **collagenase**; we recommend 3 minutes at most, even with lower amounts of collagenase.

• **Density**: The cultures must be maintained at a high density (200+ colonies in a 60 mm dish).

• hESCs grown in culture are always under selection pressure of **proliferation vs. differentiation**. The cultures should be fed every day; do not exhaust medium by not feeding. Scrape clearly differentiated areas out with a 21½ gauge needle.

• Cells can be transferred from MEF-CM right into this medium, without prior adaptation

ISCI2/SOP/020 – Passage of hES cells (MEDIA 10, Control)

Purpose:

To define the ISCI2 standard culture conditions for the subculture of human embryonic stem (hES) cells.

Items required:

- Collagenase – see Appendix 1, or suitable alternative
- hES medium – see Appendix 1
- PBS (Ca^{2+}, Mg^{2+} free)
- Prepared feeder cells, MEF or suitable alternative
- $25cm^3$ Culure flasks
- Pipettes, 5ml and 10ml
- Class II Microbiological Safety Cabinet (MSC)
- Cell culture microscope
- 15ml centrifuge tubes
- Centrifuge (refrigerated)
- 3mm diameter sterilised glass beads (Phillip Harris Scientific, Cat No. G50-203) or equivalent
- CO_2 incubator
- Glass beads

Notes:

- This protocol assumes that hES cells are grown in $25cm^2$ flasks. Other culture vessels may be used; however the volume of medium per vessel may need to be adjusted.

Procedure:

1. Using a microscope, select hES cells that are ready to passage.

2. Transfer flask to a Class II MSC and aspirate the spent medium from the flask.

3. Add 2ml of collagenase per 25cm^2 flask. Incubate the flask at 37°C for 8-10 minutes, until the edges of the hES colonies start to curl (as observed under the microscope).

4. Gently scrape the cells from the flask by addition of 10-20 glass beads, which are then rolled across the surface of the flask.

5. Add 5ml hES medium and gently resuspend the cells.

6. Transfer suspension to a centrifuge tube. Centrifuge at 50 x g for 3 minutes at 4°C.

7. Whilst the hES cells are spinning, use a plastic pipette to remove the spent medium from the required number of flasks of feeder cells (i.e. for a 1:4 split of hES cells, 4 flasks of feeders are required), and wash the feeders once with PBS.

8. Using a plastic pipette, remove the supernatant from the hES cell pellet.

9. Gently flick the tube to disperse the pellet, and carefully resuspend the cells in an appropriate volume of hES medium, allowing 1ml of medium for each new culture to be set up (i.e. for a 1:4 split ratio, resuspend in 4ml of medium).

10. Using a plastic pipette, remove the PBS from the feeder layers.

11. Add 1ml of hES cell suspension to each flask of feeders.

12. Add a further 4ml of hES medium per flask, and then carefully transfer to a 37°C incubator, maintaining an even distribution of cells across the feeder layer.

13. Microscopically check the appearance of the cells each day, and feed with fresh hES medium daily, until the cells are ready to passage.

Composition of Specific 'Test' Media

MEDIA 1 (LI ET. AL., 2005)

Composition

Composition				
Component	**Supplier**	**Cat #**	**Stock Aliquot**	**Final**
XVIVO-10	Lonza		100%	100%
L-Glutamine	Invitrogen	25030-081		2mM
β-mercaptoethanol	Invitrogen	21985-023	4mg/ml	0.1mM
Non-essential Amino acids	Invitrogen	11140-050		1%
bFGF	RND Systems	3718-FB/CF	2µg/ml	40ng/ml
hFLT3	RND Systems	308-FKN/CF	2.5µg/ml	15ng/ml

1. Add 10ml PBS (Ca^{2+}, Mg^{2+} free) to 0.292g L-glutamine in a 15ml tube.
2. Add 7µl of β-mercaptoethanol to the L-glutamine/PBS and mix well.
3. Into a 225ml 0.2 micron cellulose acetate filtering unit add:
   - 100 ml XVIVO-10
   - 1ml L-Glutamine/ β-mercaptoethanol solution
   - 1ml 100X non-essential amino acid solution
4. Filter.

Store at 4°C and use within two weeks

5. Add just before passaging bFGF to 40ng/ml and hFLT3 to 15ng/ml

To 10 ml of Media 1 add

- 200µl of 2µg/ml bFGF stock.
- 60µl of 2.5µg/ml hFLT3 stock

MEDIA 2 (LIU ET. AL., 2006)

Composition

Composition				
Component	**Supplier**	**Cat #**	**Stock Aliquot**	**Final**
DMEM/F12	Invitrogen	21041-025	100%	100%
L-Glutamine	Invitrogen	25030-081		1mM
N2	Invitrogen	17502-048	100x	1x
B27	Invitrogen	17504-044	50x	1x
β-mercaptoethanol	Invitrogen	21985-023	4mg/ml	0.1mM
Non-essential Amino acids	Invitrogen	11140-050		1%
bFGF	RND Systems	3718-FB/CF	2µg/ml*	100ng/ml

1. Add 10ml PBS (Ca^{2+}, Mg^{2+} free) to 0.146g L-glutamine in a 15ml tube

2. Add 7µl of β-mercaptoethanol to the L-glutamine/PBS and mix well.

3. Into a 225ml 0.2 micron cellulose acetate filtering unit add:
   - 100 ml DMEM/F12

- 1ml L-Glutamine/ β-mercaptoethanol solution
- 1ml 100X non-essential amino acid solution
- 1ml N2 supplement
- 2ml B27 supplement

1. Filter.

Store at 4°C and use within two weeks

4. Add just before passaging bFGF to 100ng/ml

To 10 ml of Media 2 add

- 500µl of 2µg/ml bFGF stock.

MEDIA 3 (VALLIER ET. AL., 2005)

Composition				
Component	**Supplier**	**Cat #**	**Stock Aliquot**	**Final**
IMDM	Invitrogen	21980-32	100%	50%
F12	Invitrogen	31765-027	100%	50%
Insulin	Invitrogen	1285-014	4mg/ml	7µg/ml
L-Glutamine	Invitrogen	25030-081		4mM
Transferrin	Invitrogen	11105-012		15µg/ml
Monothioglycerol	Sigma-Aldrich	M6145		450µM
BSA	Sigma-Aldrich Europa bioproducts	A-1470 EQBAC62 (lot BAC62-624		5mg/ml
Lipid	Invitrogen	11905-031	100X	1%
Activin A	RND Systems	338-AC/CF	2µg/ml*	10ng/ml
bFGF	RND Systems	3718-FB/CF	2µg/ml*	12/ng/ml

***These are supplied to the consortium as single vials and will be aliquoted 2µg/ml working stock solutions.**

To make 500mls.

IMDM (Invitrogen 21980-32): 250ml (50%)

F12 (Invitrogen 31765-027): 250 ml (50%)

L-Glutamine ?/

BSA (Sigma A-1470 or Europa bioproducts EQBAC62 lot BAC62-624): 2.5gr (5mg/ml) Lipid 100X (Invitrogen 11905-031): 5ml (1%)

Monothioglycerol (Sigma M6145): 20 µl (450 µM)

Insulin 4mg/ml: 875 µl (7µg/ml)

Transferin 30 mg/ml: 250µl(15µg/ml)

MEDIA 4 (LU ET. AL., 2005)

Composition

Composition				
Component	**Supplier**	**Cat #**	**Stock Aliquot**	**Final**
DMEM/F12	Invitrogen	21041-025	100%	100%
Insulin	Invitrogen	1285-014	4mg/ml	160µg/ml
L-Glutamine	Invitrogen	25030-081		1mM
Transferrin	Invitrogen	11105-012		88µg/ml
BSA (Fraction V)	Invitrogen	15260-037	7.5%	2.5mg/ml
Cholesterol Supp.	Invitrogen	12531-018	250x	2.5x
bFGF	RND Systems	3718-FB/CF	2µg/ml*	4ng/ml
Wnt3a	RND Systems	1324-WN/CF	2µg/ml*	100ng/ml
BAFF	Invitrogen	PHC1674	20µg	100ng/ml

1. Into a 225ml 0.2 micron cellulose acetate filtering unit add:
    - 100 ml DMEM/F12
    - BSA (fraction V) ??
    - 1ml Cholesterol Supplement
    - 4ml Insulin
    - xx Transferrin
    - BSA (fraction V)

2. Filter.

Store at 4°C and use within two weeks

5. Add just before passaging bFGF to 4ng/ml, WNT3a to 100ng/ml and BAFF to 100ng/ml

To 10 ml of Media 4 add

- 2µl of 2µg/ml bFGF stock.
- 500µl of 2µg/ml WNT3a stock
- 500µl of 2µl/ml BAFF stack

MEDIA 5 (YAO ET. AL., 2006)

Composition

Composition				
Component	**Supplier**	**Cat #**	**Stock Aliquot**	**Final**
DMEM/F12	Invitrogen	21041-025	100%	100%
L-Glutamine	Invitrogen	25030-081		2mM
N2	Invitrogen	17502-048	100x	1x
B27	Invitrogen	17504-044	50x	1x
β-mercaptoethanol	Invitrogen	21985-023	4mg/ml	0.11mM
NEAA	Invitrogen	11140-050		1mM
BSA (Fraction V)	Invitrogen	15260-037	7.5%	0.5mg/ml
bFGF	RND Systems	3718-FB/CF	2µg/ml*	20ng/ml

1. Add 10ml PBS (Ca^{2+}, Mg^{2+} free) to 0.292g L-glutamine in a 15ml tube

2. Add 7µl of β-mercaptoethanol to the L-glutamine/PBS and mix well.

3. Into a 225ml 0.2 micron cellulose acetate filtering unit add:
   - 100 ml DMEM/F12
   - 1ml L-Glutamine/ β-mercaptoethanol solution

- 1ml 100X non-essential amino acid solution
- 1ml N2 supplement
- 2ml B27 supplement
- BSA (fraction V) ??

3. Filter.

Store at 4°C and use within two weeks

5. Add just before passaging bFGF to 20ng/ml

To 10 ml of Media 5 add

- 100µl of 2µg/ml bFGF stock.

MEDIA 6 (ITOSHI)

Composition: N/A

MEDIA 7 (MTESR)

Composition: N/A

MEDIA 8 (STEM-PRO)

Composition

Description	Cat.	no. Size	Storage	Shelf Life
STEMPRO® hESC SFM	A10007-01	1 kit		
Contains:				
DMEM/F-12 with GlutaMAX	10565-018	500 mL	2 to 8°C	12 months
			(protect from light)	
STEMPRO® hESC Supplement	A10006-01	10 mL	-5 to -20°C	12 months
			(in the dark)	
Bovine Serum Albumin 25% (BSA)	A10008-01	40 mL	2 to 8°C	12 months
			(protect from light)	

Notes

Storage and Handling:

• Supplement is supplied as a frozen sample. Thaw supplement prior to use, re-freeze in desired volumes, and store them immediately at -20°C. *See Media Preparation.*

• **Avoid multiple** freeze thaw cycles of supplement.

• Thawed STEMPRO® hESC SFM Growth Supplement must be stored at 2-8°C (Stable up to 1 week)

• Reconstitute minimal (daily) volumes of working culture medium to 1X, prior to use.

• Reconstituted 1X culture media is stable only for 24 hours and we strongly recommend to pre-equilibrate to temperature and gases.

Media Preparation

Wash medium: Add BSA 25% at a final concentration of 0.1% to D-MEM/F-12.

Complete medium: Thaw supplement in 37°C water bath (minimize dwell time), and prepare according to the chart:

Note: If not used immediately, store at 2°C to 8°C in the dark. Use the same day.

MEDIA 10 (CONTROL)

Composition

DMEM/F12	80%
GIBCO knockout SR	20%
Non-essential amino acids	1%
L-glutamine	1mM
β-mercaptoethanol	0.1mM
human bFGF	4ng/ml

Formulation

Standard hES Medium.

4. Add 10ml PBS (Ca^{2+}, Mg^{2+} free) to 0.146g L-glutamine in a 15ml tube.
5. Add 7µl of β-mercaptoethanol to the L-glutamine/PBS and mix well.
6. Into a 225ml 0.2 micron cellulose acetate filtering unit add:
   - 80ml DMEM/F12
   - 20ml Knockout SR.
   - 1ml L-Glutamine/ β-mercaptoethanol solution
   - 1ml 100X non-essential amino acid solution
   - 200µl of 2µg/ml bFGF stock.
   - Antibiotics (optional, e.g. Gentamicin)
7. Filter.
8. Store at 4°C and use within two weeks.

Notes:

- Human bFGF: dissolve 10µg in 5ml of 0.1% BSA in PBS (Ca^{2+}, Mg^{2+} free).Aliquot and store at -20°C.

Aliquot SR and store at -20°C; do not repeatedly freeze and thaw